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Control of Growth and Squamous Differentiation in Normal Human Bronchial Epithelial Cells by Chemical and Biological Modifiers and Transferred Genes

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The majority of human lung cancers arise from bronchial epithelial cells. The normal pseudostratified bronchial epithelium is composed of basal, mucous, and ciliated cells. This multidifferentiated epithelium usually responds to xenobiotics and physical injury by undergoing basal cell hyperplasia, mucous cell hyperplasia, and squamous metaplasia. One step of the multistage process of carcinogenesis is thought to involve aberrations in control of the squamous metaplastic processes. Decreased responsiveness to regulators of terminal squamous differentiation may confer a selective clonal expansion advantage to an initiated cell. We studied the effects of endogenous [e.g., transforming growth factor β_1 (TGF-β_1) and serum] and exogenous [e.g., 12-*O*-tetradecanoyl-13-phorbolacetate (TPA), tobacco smoke condensate, and aldehydes] modifiers of normal human bronchial epithelial (NHBE) cell in a serum-free culture system. NHBE cells are growth inhibited by all of these compounds and induced to undergo squamous differentiation by TGF-β_1 or TPA. In contrast, lung carcinoma cell lines are relatively resistant to inducers of terminal squamous differentiation which may provide them with a selective growth advantage. Chemical agents and activated protooncogenes (*ras,raf,myc*) altered the response to endogenous and exogenous inducers of squamous differentiation and caused extended cellular lifespan, aneuploidy, and/or tumorigenicity. The data suggest a close relationship between dysregulation of terminal differentiation pathways and neoplastic transformation of human bronchial epithelial cells.

Introduction

Proliferation and terminal differentiation of normal cells are usually controlled by different signal transduction pathways. The interactions of these two controlling mechanisms ensure the homeostatic balance between cell proliferation and terminal differentiation. Uncoupling of the normal balance between the two interacting systems due to either genetic or epigenetic changes is thought to be a critical step in the generation and further progression of a malignant cell phenotype. It has been suggested that squamous metaplasia and dysplasia play an important role in lining epithelial cell transformation and that preneoplastic cells acquire a selective resistance to inducers of differentiation because the controlling pathways for the terminal aspects of this cellular process are defective. As a consequence, they may have a clonal growth advantage over normal cells (*1-4*) and, in agreement with this hypothesis, diminished response to inducers of differentiation is a common characteristic of carcinomas (*1,5-11*).

Constitutive production of mitogens (autocrine growth control) (*12,13*), reduced or ablated dependence on paracrine growth factors, selective resistance to cytotoxic agents, as well as escape from cell-cell communication, are other conceivable aberrations that may generate selective clonal expansion of preneoplastic and neoplastic cells. To gain an understanding of mechanisms involved in human carcinogenesis, it is critical to analyze the interactions of the multiple pathways controlling growth and differentiation of the epithelial cells. In this review, we will discuss the use of cultured human bronchial epithelial cells as a model system to investigate the molecular and cellular mechanisms controlling growth and differentiation of epithelial cells.

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Activated proto-oncogenes that have been associated with lung cancer include the *ras*, *myc*, ard *raf* families (*14–18*), and transfected oncogenes have been shown to transform normal human epithelial cells (*19*). Very recently, we have isolated a SV40 T-antigen containing bronchial epithelial cell culture that has an indefinite lifespan (*20*) but retains the ability to respond to certain inducers of squamous differentiation (*21*). Because the differential response of normal and transformed epithelial cells to inducers of differentiation can be employed to determine aberrations of tumor cells in pathways controlling proliferation (*5,7–10*), we will also review the changes in the differentiation program caused by transfected oncogenes.

Serum-Free Culture System for NHBE Cells

Serum-free culture systems have been developed for human and mouse keratinocytes (*22–24*) and for normal (*25–27*) and neoplastic epithelial cells (*28–30*). Serum-free media permit studies of individual elements controlling growth and differentiation in a well-characterized environment. The application of serum-free media identified other components that had been masked by serum supplementation in culture medium and allowed the growth of some cell types that cannot be duplicated in conventional, serum-containing media. In our system, serum was found to inhibit growth of normal human bronchial epithelial (NHBE) cells by inducing terminal squamous differentiation (*7,25,31*). Therefore, our studies have been performed using a serum-free culture system developed from medium MCDB 151, optimized for osmolarity and $[Ca^{2+}]$, and supplemented with insulin (5 μg/mL), epithelial growth factor (EGF) (5 ng/mL), transferrin (10 μg/mL), hydrocortisone (0.2 μM), bovine pituitary extract, gentamicin (50 μg/mL), and trace elements (*25*).

Endogenous Inducers of Squamous Differentiation of NHBE Cells

Although most carcinogenesis studies have been focused on the activation of proliferative responses by growth factors and oncogenes, it is equally important to analyze defects in mechanisms regulating terminal differentiation. In agreement, the diminished response to inducers of terminal differentiation is a phenotypic *in vitro* marker of neoplastic epithelial cells (*6–8,11,32*). Bronchial epithelial cells undergo several types of differentiation, i.e., ciliary, mucous, and squamous. Squamous differentiation is characterized by squamous morphology, irreversible cessation of DNA synthesis and cell division, continued RNA synthesis (for 3 days), formation of cross-linked envelopes (CLE), and enhanced release of plasminogen activator (PA).

TGF-β_1 Induces Squamous Differentiation

Transforming growth factor (TGF-β_1) expression and TGF-β_1-specific receptors have been detected in a variety of normal and tumor cells (*33–36*). The cellular effects reported for TGF-β_1 are very complex (*37*). TGF-β_1 is growth inhibitory for normal and certain epithelial tumor cell lines (*38–40*), stimulates proliferation of fibroblasts (*41*), but also exhibits differential effects on proliferation of normal and malignant cells (*42,43*). Aberrations in pathways controlled by TGF-β_1 in a negative manner can be expected to cause uncontrolled cell proliferation.

In our model system, insulin, EGF, retinoic acid, undefined pituitary extract constituents, and epinephrine stimulate cell multiplication (*25*), whereas serum, high concentration of extracellular Ca^{2+}, and TGF-β_1 are inducers of terminal squamous differentiation of NHBE cells (Table 1) (*7,25,42*). TGF-β_1 inhibits the clonal growth rate and DNA synthesis of NHBE cells in a dose-dependent manner, i.e., 4 pM TGF-β_1 causes a rapid 95% inhibition of DNA synthesis, but RNA synthesis remained at near normal rates for 3 days (*42*). The DNA synthesis inhibition is irreversible and accompanied by an increase of CLE, PA release, and cell surface area. The IgG fraction of rabbit TGF-β_1 antiserum neutralizes both the inhibition of DNA synthesis caused by TGF-β_1 or serum and suppressed the squamous morphology. The data implied that TGF-β_1 is an important differentiation-inducing serum factor in NHBE cells. Similar findings have been reported for rat hepatocytes (*44*), mouse or human epiderminal keratinocytes (*24,45*), and rabbit tracheal epithelial cells (*46,47*).

In addition, the latent form of TGF-β_1 is detected in conditioned medium of NHBE cultures (*48*). The isolation of a TGF-β_1 binding protein (α_2M) in serum has led to the speculation that secreted TGF-β_1 is protected by formation of a TGF-β_1-protein complex and released at target sites (*49*). However, the precise activation mechanism of the latent form of TGF-β_1 is not known. Furthermore, the induction of squamous differentiation by TGF-β_1 and serum is a density-dependent process and high density cul-

Table 1. Endogenous and exogenous modifiers and their effects on growth and differentiation of NHBE cells.

Modifier	Growth[a]	Differentiation[a]
Endogenous agents		
Insulin	I	NE
EGF	I	NE
Pituitary extract	I[b]	NE
Epinephrine	I[b]	D
Retinoic acid	I	NE
Ca^{2+}	I[c]	I[d]
TGF-β_1	D	I
Serum	D	I
Exogenous agents		
TPA	D	I
Teleociden B	D	I
Cigarette smoke condensate	D	I
Aldehydes	D	I

[a] I, increase; D, decrease; NE, no effect.
[b] Only in presence of EGF.
[c] In low density culture.
[d] In confluent cultures.

tures of NHBE cells, i.e., > 11,000 cells/cm^2 culture surface, are more resistant to either agent (J. F. Lechner and A. M. A. Pfeifer, unpublished observation). This suggests the presence of a putative endogenous factor(s) that is characterized by antagonism to TGF-β_1 or serum and is present in a sufficient amount only in high density cultures (Fig. 1).

The Role of Ca^{2+} in Controlling Squamous Differentiation of NHBE Cells

The role of Ca^{2+} in regulating epithelial cell proliferation and differentiation has been delineated in a number of systems. Rat tracheal epithelial cells (*50*) and human keratinocytes (*51*) show maximal growth at 0.8 mM and 0.3 mM Ca^{2+}, respectively. Mouse keratinocytes (*52*), human mammary epithelial cells (*53*), and various other murine and human cells proliferate in low Ca^{2+} (0.03–0.07 mM) (*54*) and undergo terminal differentiation in high Ca^{2+}.

In experiments comparing clonal and high density cultures of NHBE cells in serum-free medium, the effect of extracellular Ca^{2+} is dependent on the number of cells per square centimeter. Up to 1 mM Ca^{2+} is not growth-inhibiting at clonal density (*25*), whereas high density cultures (> 11,000 cells/cm^2) are induced to undergo squamous differentiation by 0.3 mM Ca^{2+} and above. However, squamous differentiation of clonal density cultures of NHBE cells can be induced by Ca^{2+} in the presence of complementary undefined factors. Thus, in combination with low amounts of fetal bovine serum (1.25%), Ca^{2+} causes clonal density cultures of NHBE cells to undergo squamous differentiation in a dose-dependent manner (Fig. 1) (*31*). The divergent findings in low versus high density cultures are consistent with the hypothesis that NHBE cells produce a differentiation-inducing autogenous factor that accumulates to an effective concentration only in high density cultures and is complemented by extracellular Ca^{2+}. The synergistic effect of Ca^{2+} suggests either an increased release of the factor or an activation of the factor by Ca^{2+}.

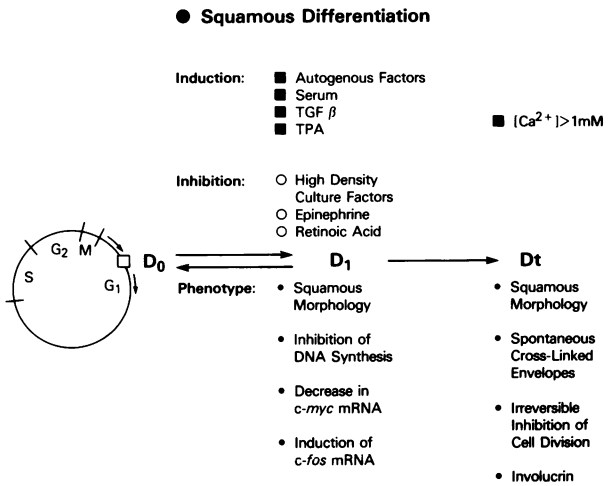

FIGURE 1. Model of the multistage squamous differentiation of human bronchial epithelial cells. Cells at D_0 or D_1 (nonterminally differentiation state) can re-enter proliferation or undergo terminal differentiation (Dt). Ca^{2+} induces Dt only in the presence of serum or autogenous factors expressed at cell confluence.

Recently, we investigated the question of whether the effect of differentiation inducers, [e.g., TGF-β_1, serum, or 12-*O*-tetradecanoyl-13-phorbolacetate (TPA) is mediated by an increase of free cytosolic calcium ion ($[Ca^{2+}]_i$), employing a fluorescence assay with fura-2. Fura-2 forms a fluorescent calcium complex and the emitted flourescence at 510 nm is calcium ion-concentration dependent (*55*). We found that serum caused a 2-fold enhancement of $[Ca^{2+}]_i$ flux into the cytosol, whereas neither TGF-β_1 nor TPA changed cytosolic $[Ca^{2+}]_i$ concentration (*55*). Thus, early and rapid changes in intracellular $[Ca^{2+}]_i$ are not absolutely required for the cells in order to undergo squamous differentiation.

Squamous Differentiation Induction by TPA and Its Possible Mechanism

The effects of TPA in the mouse skin carcinogenesis model suggest that two different classes of compounds (initiators and promoters) are involved in oncogenesis and further, that carcinogenesis is a multistep process (*2*). In addition to their promoting activity on mouse epidermis, certain phorbol esters have multiple effects on growth and squamous differentiation of cells *in vitro*. In most types of epithelial cells, the predominant response to TPA is growth inhibition and/or differentiation (*56–60*), whereas some fibroblast systems are mitotically stimulated by TPA (*61,62*).

Despite their different chemical structures, the tumor promoters TPA, teleocidin B (indol alkaloid), aplysiatoxin, debromoplysiatoxin, and hydrogen and benzoyl peroxides induce terminal squamous differentiation of NHBE cells (*59,63,64*). There is evidence from other systems that the induction of squamous differentiation by TPA and related compounds is mediated by the direct activation of protein kinase C (*65,66*), which is the specific membrane receptor for TPA (*67*) and teleocidin B (*68*). In human keratinocytes, TPA causes terminal squamous differentiation (*56,57,69*), and the event is dissociated from the TPA-induced phospholipase A_2 activation, including the subsequent release of arachidonic acid and induction of prostaglandin synthesis (*70*). The biosynthesis of prostaglandins and hydroxyarachidonic acid involves the intermediate formation of unstable hydroxyperoxy derivatives and concomitant release of active oxygen (*71*). Generation of active oxygen species leading to lipid peroxidation and release of calcium ion into the cytosol has been described for tumor promoters, i.e., TPA, benzoyl peroxide, and hydrogen peroxide (*71*). We have found, however, that the induction of squamous differentiation of NHBE cells by TPA does not involve free oxygen radicals (*72*). Thus, the activation of phospholipase A_2 and lipid peroxidation are presumably not essential for this effect of TPA on NHBE cells. This is consistent with our observation that free cytosolic $[Ca^{2+}]_i$ is unaltered after exposure of NHBE cells to TPA (*55*). Moreover, TPA pretreatment of

NHBE cells resulted in a diminished $[Ca^{2+}]_i$ response induced by serum (*55*). However, benzoyl peroxide or hydrogen peroxide do not bind to protein kinase C (*73*) and are potent inducers of lipid peroxidation associated with the disturbance of $[Ca^{2+}]_i$ homeostasis (*71*). Therefore, lipid peroxidation may play a role for this class of tumor promoters. In conclusion, various types of tumor promoters induce squamous differentiation of NHBE cells by different mechanisms of action.

Cigarette Smoke Condensate Induces Features of Terminal Squamous Differentiation

Because tobacco smoke is the major cause of lung cancer, we have been studying the effects of tobacco smoke and its components on growth and differentiation of NHBE cells (*74*). The effects of whole tobacco smoke condensate and individual fractions on clonal growth rate, plasminogen activity, CLE formation, ornithine decarboxylase activity (ODC), EGF binding, thiol depletion and DNA single strand breaks (SSB) (*75*) have been investigated. Cigarette smoke condensate (CSC), the two basic fractions (B1a and B1b), the weakly acidic fraction (WAe), and the neutral methanol fraction (Nmeoh) are growth inhibitory and induce CLE formation and squamous morphology. Neither CSC nor any of the fractions significantly affect intracellular thiol levels or ODC activity. DNA SSB were detected with 100 μg/mL CSC and low concentrations of Nmeoh (5 μg/mL). Plasminogen activator was increased by Nmeoh only. CSC, WAe and Nmeoh caused a decrease in EGF binding that was primarily related to a diminished EGF receptor affinity (*75*).

Because TPA neutralizes the growth stimulation induced by EGF in prostatic epithelial cells (*76*) and induces markers of differentiation in NHBE cells (*59*), we investigated the possibility of CSC and particularly Nmeoh having a TPA-like effect on growth of NHBE cells. CSC or Nmeoh, but not B1a or B1b, inhibited the binding of phorbol dibutyrate to a small degree (*77*), indicating the presence of a low concentration of TPA-related compounds in CSC and that the majority of tumor promoting components in CSC are different from TPA. However, subsequently, we have found that TPA induces interleukin-1 mRNA synthesis in NHBE cells but CSC does not, confirming the suggestion that the concentration of TPA-like substances in CSC must be extremely low (B. Gerwin and M. Miyashita, unpublished data).

Effects of Tobacco Smoke-Related Aldehydes on Squamous Differentiation of NHBE Cells

As aldehydes have been found in the gaseous phase of tobacco smoke and are mutagenic (*78*), cytotoxic (*79,80*), and tumor promoting, we have been studying the effects of various aldehydes on growth and differentiation of cultured bronchial epithelium (*78*). Clonal growth rate, squamous differentiation, DNA damage, DNA synthesis, arachidonic acid and choline release, the activity of aryl hydrocarbon hydroxylase (AHH), and ODC were measured. Formaldehyde, acetaldehyde, and acrolein inhibit growth and induce CLE formation (Table 1) (*63,80*), but only acetaldehyde increases squamous morphology (*78,79*). Formaldehyde causes DNA single strand breaks and DNA-protein cross-links and dramatically enhances ODC activity. The inhibitory effect of formaldehyde or acetaldehyde on AHH, DNA and RNA synthesis, as well as the release of arachidonic acid and choline is similar for both compounds. Thus, aldehydes have complex effects on NHBE cells and share with TPA and serum the ability to increase the number of CLE-competent cells.

Thiol levels are decreased in NHBE cells and cultured human bronchial fibroblasts following the exposure to acrolein, whereas formaldehyde and acetaldehyde are much less effective (*78,80*). Since the depletion of GSH by acrolein is not correlated with an increase in GSSG (*81*), the generation of radicals and subsequent lipid peroxidation may not play a role for effects of aldehydes on squamous differentiation of NHBE cells. However, it is possible that free cytosolic $[Ca^{2+}]_i$ is involved in the induction of CLE formation because aldehydes increase the cytosolic $[Ca^{2+}]_i$ concentration (*82*) and CLE formation is a Ca^{2+}-dependent mechanism (*83*).

Differential Response of Lung Carcinoma Cells to Inducers of Squamous Differentiation

Resistance to inducers of terminal differentiation has been identified as a common marker for malignant cells. For example, only transformed mouse keratinocytes formed foci of cells resistant to high extracellular Ca^{2+} in culture, and the number of foci is elevated by TPA (*1*). This observation is consistent with the theory that initiated cells are selectively resistant to terminal differentiation by tumor promoters and thus, acquire a selective clonal growth advantage (*1,2*). Similar effects have been reported for mouse keratinocytes initiated *in vivo* or *in vitro* with 7,12-dimethylbenz(a)anthracene or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*84*) and for rat liver cells transformed *in vitro* by aflatoxin B_1 which become resistant to extracellular Ca^{2+} and TGF-β_1, respectively (*43*).

We examined the responsiveness of several lung carcinoma cell lines of different histopathology (A 549, Calu 1, Hut 292) to inducers of terminal squamous differentiation in NHBE cells. None of the tumor cell lines underwent squamous differentiation induced by serum (*7,48*), TGF-β_1 (*42*), or TPA (*64*). Moreover, lung carcinoma cell lines are growth inhibited in serum-free medium, and growth is stimulated by serum, indicating that they acquired the ability to respond to unidentified growth factors in serum. In addition, CSC or its fractions did not cause significant morphological changes in carcinoma cell lines (*77*) toward a squamous differentiated phenotype. The resistance of tumor cell lines to inducers of terminal squamous differentiation is not due to detectable changes in TPA membrane receptors (*42*). Assays analyzing the properties of the TGF-β_1-specific receptors showed no significant differ-

ences for A549 and Calu 1 compared to NHBE cells, whereas a third line, Hut 292, possessed a smaller number of receptors with high affinity to TGF-β_1 (*42,85*). The differential response of the carcinoma cell lines to inducers of differentiation implies the selective clonal expansion advantage of neoplastic cells generated by an imbalance between growth- and differentiation-controlling pathways.

Role of cAMP in Squamous Differentiation of NHBE Cells

We have identified several endogenous and exogenous inducers of squamous differentiation in NHBE cells, and it was tempting to search for a common mediator in their pathways. We previously reported that the growth rate of NHBE cells is enhanced by epinephrine or other cAMP enhancers, i.e., 3-isobutyl-1-methyl-xanthine, dibutyryl AMP, and cholera toxin, in the presence of EGF and pituitary extract (*86*). The growth stimulation is accompanied by an increase of cAMP and ODC activity (*86,87*), however, elevated ODC activity is necessary but not sufficient for stimulation of NHBE growth (*86*). The effect of cAMP can either result in stimulation or reduction of proliferation in epithelial cells depending on the location of cAMP synthesis in the cell and the type of cAMP-dependent protein kinase present (*88*).

In our system, cholera toxin, a stimulator of intracellular cAMP, antagonizes the induction of terminal squamous differentiation by serum (*48*), which indicates a negative correlation between cAMP content and induction of differentiation. To test this hypothesis, we examined the effect of epinephrine, cholera toxin, and pertussis toxin on growth of NHBE cells exposed to TGF-β_1. We showed that epinephrine neutralizes the effect of TGF-β_1 (Table 1) (*42*), whereas growth stimulation caused by epinephrine is antagonized by TGF-β_1. Thus, epinephrine and TGF-β_1 are mutually antagonistic. The epinephrine effect is associated with an increase in cAMP, but TGF-β_1 antagonizes epinephrine without altering intracellular cAMP levels. The antagonism between TGF-β_1 and epinephrine does not involve changes at the receptor level, as the characteristics of the TGF-β_1 receptor are unaltered by epinephrine (*36*). Therefore, the antagonism of TGF-β_1, and epinephrine does not reflect a common pathway. It is more likely that these compounds affect different mechanisms implicated in control of growth and differentiation of NHBE cells. However, since epinephrine also partially antagonizes TPA (T. Masui, unpublished results), TGF-β_1 and TPA pathways to squamous differentiation seemingly converge prior to passing beyond the irreversible step.

Functions of c-*myc*, c-*fos*, and c-Ha-*ras* Protooncogenes in the Differentiation Process of NHBE Cells

To date, more than 30 oncogenes have been identified and associated with animal or human tumors (*89,90*). The normal cellular homologues (protooncogenes) are characterized by unique features suggesting an essential physiological role of these genes in the regulation of cell proliferation and differentiation: a) protooncogenes have been highly conserved through evolution; b) the gene products are specifically located in the nucleus, cytoplasm, or bound to the plasma membrane; c) the corresponding proteins exhibit distinctive functional activities; and d) several protooncogenes resemble growth factors or growth factor receptors (*91–94*). The fact that protooncogenes are differentially expressed during embryogenesis (*95*) and show stage-specific expression during human monocyte differentiation (*96*) also suggests a role for these cellular genes in induction of differentiation. Along these lines, c-*src* expression has been mostly correlated with differentiation of neuronal cells, c-*fos* with hematopoietic differentiation, and c-*fms* with macrophage differentiation (*97*). The expression of c-*myc* and c-*myb* is negatively correlated to erythroid and neuronal differentiation and is dependent on the cell lineage and on the differentiation stage (*97*). Moreover, c-*ras* proteins are presumably involved in differentiation of various cell types (*98,99*).

We investigated the association of c-*fos*, c-*myc*, and c-Ha-*ras* genes with differentiation of NHBE cells employing TGF-β_1 or TPA as differentiation-inducing agents and epinephrine as an antagonizer of TGF-β_1. c-*fos* mRNA steady-state levels are increased 2-fold within 1 hr after TGF-β_1 exposure and came back to normal after 3 hr (T. Masui, unpublished observations). The simultaneous application of TGF-β_1 and epinephrine caused up to 5-fold induction of c-*fos* mRNA after 1 hr. In contrast to c-*fos*, c-Ha-*ras* mRNA steady-state levels were hardly affected by TGF-β_1; c-*myc* mRNA decreased in a dose-dependent manner to 40 to 80% of the original value. The effect on c-*myc* mRNA was transient for low concentrations of TGF-β_1, but resulted in an irreversible reduction of c-*myc* mRNA at a high TGF-β_1 dose (100 pg/mg). Epinephrine neutralized the effect of TGF-β_1 on c-*myc* expression. TPA caused the same pattern of changes in protooncogene mRNA steady-state levels characterized by an increase of c-*fos*, no change of c-Ha-*ras*, and a reduction in c-*myc* during the first hour.

Based on our experiments, we cannot discriminate if the c-*fos* induction is a causative event for differentiation or if its function is only signal transduction. However, since epinephrine inhibits terminal squamous differentiation of NHBE cells by TGF-β_1, but induces synergistically c-*fos* mRNA, the c-*fos* signal appears to be associated with signal transduction rather than induction of squamous differentiation *per se*. In accordance, two important cellular signal transduction pathways, the protein kinase C/Ca^{2+} and the cAMP system, have been associated with c-*fos* induction (*100–102*). In PC 12 cells, cAMP or activation of the protein kinase C (PKC) accompanied by the increase of free cytosolic $[Ca^{2+}]_i$ cause a rapid induction of c-*fos* mRNA (*101*). However, c-*fos* is stimulated in fibroblasts by the PKC/Ca^{2+} system but not by cyclic purine nucleotides (*102*). As previously discussed in this review, neither TGF-β_1 nor TPA alter the free cytosolic $[Ca^{2+}]_i$ concentration in NHBE cells, and cAMP levels are not af-

fected by TGF-β_1, suggesting the involvement of another pathway, or pathways, which are not controlled by either free cytosolic $[Ca^{2+}]_i$ or cAMP.

An inverse correlation of c-*myc*, N-*myc*, or c-*myb* expression has been documented during HL 60 differentiation (*97*), during *in vitro* differentiation of neuroblastoma cell lines (*103*), and during induction of differentiation of F9 teratocarcinoma cells, respectively (*104*). c-*myc* has been implicated in the regulation of cellular proliferation, and downregulation of c-*myc* mRNA may be a consequence for or may trigger differentiation in certain cell systems (*105*).

The irreversible downregulation of c-*myc* mRNA in NHBE cells exposed to high levels of TGF-β_1 supports the first mechanism but cannot exclude a function of the c-*myc* gene for mediating differentiation signals. However, since epinephrine neutralizes the effects of TGF-β_1 on inhibition of growth, as well as on c-*myc* expression, the reduction in c-*myc* expression may represent only a blockage of proliferation rather than a differentiation-inducing signal.

In conclusion, alterations in the expression of c-*myc*, c-*fos*, and c-Ha-*ras* occur parallel with the induction of squamous differentiation in NHBE cells. The findings to date imply a function of these protooncogenes in signal transduction rather than in signal induction. Further studies on protooncogene expression under the influence of agents antagonizing terminal squamous differentiation are required to determine the precise role of these genes. Transfection experiments in which c-*fos*, c-*myc*, and c-Ha-*ras* sequences are being introduced into normal or immortalized human bronchial epithelial cells will provide additional information on the role of these protooncogenes in induction of squamous differentiation.

Carcinogenesis Studies on NHBE Cells with Nickel Sulfate

Inhalation of nickel compounds has been implicated in an increased human risk of respiratory cancer (*106*), and nickel sulfate has been shown to induce transformation of rodent cells (*107*). We tested the chronic effects of nickel sulfate on NHBE cells by exposing cultures for 40 days to a dose of 5 to 20 μg/mL nickel sulfate. These nickel sulfate concentrations resulted in a reduction of the colony-forming efficiency by 30 to 80% (*108*). After 40 days, mitoses figures appeared, and, after 75 days total incubation time, 1 focus per 10^5 cells originally inoculated arose in dishes treated with more than 5 μg/mL nickel sulfate. Most of the cell cultures produced established from nickel sulfate-transformed cells displayed increased population doublings and expressed characteristics of malignant cells in a heterogeneous manner: a) loss of sensitivity to the squamous differentiation-inducing effect of serum or TPA; b) loss of requirement of EGF for clonal growth; and c) aneuploidy and marker chromosomes.

In contrast to results reported on rodent cells (*107*), none of the nickel-transformed cell cultures was tumorigenic upon injection into athymic nude mice nor lost their anchorage dependency for growth. Studies on normal human fetal kidney epithelial cells showed that nickel sulfate induces transformation of these cells resulting in immortalization, anchorage independent growth and chromosomal aberrations. However, the presence of the v-Ha-*ras* gene is required for the expression of a fully malignant phenotype (A. Haugen, unpublished observation). Experiments are in progress to test the possibility that a second event, i.e., introduction of SV40 T-antigen gene can confer tumorigenicity to the phenotypically altered bronchial epithelial cells.

Immortalization of NHBE Cells with SV40 T-Antigen Genes

We have shown the usefulness of human bronchial epithelial cells for investigating mechanisms involved in growth and differentiation, and the effects of carcinogens and tumor promoters. However, multistage bronchial carcinogenesis studies were limited by the finite lifespan of NHBE cells. Therefore, it was one of our priorities to generate continuously growing cell lines. Other human epithelial cell types, i.e., epidermal keratinocytes, prostate, colon, bladder, have been previously immortalized by SV40 genes employing virus infection or calcium phosphate transfection (*109*). However, NHBE cells are killed by calcium phosphate. These effects could be circumvented by developing a new transfection technique in which strontium phosphate substitutes for the calcium divalent ion (*110*).

In an attempt to extend the lifespan of primary NHBE cultures, we transfected NHBE cells via strontium phosphate precipitation with a recombinant plasmid (pSV-T) containing the SV40 large T-antigen gene (BET), or by infection with either SV40 virus (BES) or an adenovirus 12/SV40 hybrid virus (BEAS) (*20*). Several colonies of morphological transformed cells were isolated and characterized (Table 2). The lifespan of the SV40-transformed cells was extended by a minimum of 20 population doublings compared to NHBE cells, but all except one of the strains eventually underwent crisis; some subsequently became established as continuously growing cell lines. The cell lines tested retained epithelial features based on electron microscopy and keratin staining but were aneuploid and expressed SV40 T-antigen measured by indirect immunofluorescence.

These various immoralized cultures were tested for tumorigenicity in nude mice. BEAS-2B revealed a very low tumorigenic potential after extended passaging. Some of the SV40-transformed clones retained the capability of NHBE cells to undergo squamous differentiation in response to serum or TGF-β^1, but all lost their responsiveness to TPA. Subclones of BEAS-2B cells were obtained that were either responsive or resistant to the differentiation, inducing effect of serum. These phenotypically very different subclones and the uniform resistance to the differentiation-inducing effect of TPA provide an excellent *in vitro* model for delineating the mechanisms of human bronchial differentiation. The apparently unlimited

Table 2. Establishment of transformed human bronchial epithelial cell cultures containing SV40 early region genes.

Designation	Method of transformation	Colonies subcultured	Number of established lines	Crisis before establishment
BEAS	Ad 12-SV40 virus	2	1	No
BES	SV40 virus	3	2	Yes
BET	RSV-T plasmid, Exp. 1	1	1	Yes
	Exp. 2	3	1	Yes

lifespan and the low tumorigenic potential of these SV40-transfected human bronchial epithelial cell lines present them as a suitable system to study the multistage bronchial carcinogenesis.

Transformation of NHBE Cells with v-Ha-*ras* and the Combination of the Oncogenes *raf* and *myc*

Three families of oncogenes, *ras*, *myc*, and *raf*, have been associated with human lung cancer. Oncogenes most frequently detected in human tumors belong to members of the *ras* gene family (Ha-, Ki-, and N-*ras*) (*111,112*). In approximately 40% of the investigated colon tumors and 50% of the tested adenocarcinomas of the lung, activated Ki-*ras* with point mutations in codon 12 was found (*17,113*). Activation of *ras* oncogenes have also been detected in lung cancer cell lines of nonsmall-cell origin, and Ki-*ras* was the predominant activated gene (*114*). Elevated levels of *ras* mRNA have mainly been encountered in squamous cell carcinomas and were normal in adenocarcinomas and small cell lung carcinomas tested (*115*). In contrast, the *myc* family (c-*myc*, N-*myc*, or L-*myc*) is commonly activated in small-cell carinoma cell lines, and amplification of *myc* has also been identified in fresh tumor tissue (*15,16,18,116*). However, a correlation between amplification of c-*myc* or N-*myc* and the appearance of a variant progressively growing phenotype has only been found in SCLC lines but not in the original tumors (*16,18,116*).

c-*raf*-1 is the cellular homolog of the viral oncogene v-*raf* (*117*) and is frequently present in normal and tumor cells of neuronal and hematopoietic origin (*118–120*). Activation of c-*raf*-1 has been implicated in various types of human tumors including gastric carcinoma (*121*), laryngeal carcinoma (*122*), renal cell carcinoma (*123*), and a lung carcinoid (*124*). In addition, overexpression of c-*raf* has been found in small cell lung carcinoma cell lines that have a characteristic deletion on the short arm of chromosome 3 (p14-23) adjacent to the location of the *raf* gene (3p25) (*125*).

To determine if *raf*, *myc*, or *ras* sequences may play an important role in human lung carcinogenesis, we introduced these oncogenes into NHBE cells. The initial study was performed with v-Ha-*ras* (plasmid H1) using a protoplast fusion technique (*19*). Under the selective pressure of 2% serum and maintenance of the cultures at confluency, four cellular foci appeared. One of them (TBE-1) was further selected in soft agar leading to TBE-1SA cell line. TBE-1 and TBE-1SA were injected into nude mice and caused tumors with a latency of 7 to 12 and 2 months, respectively. The progression of TBE-1SA in malignancy occurred concurrently with development of progressively abnormal karyotypes including aneuploidy, gaps, breaks, and exchanges (*19*), suggesting that v-Ha-*ras* may cause chromosomal abnormalities that lead to secondary alteration of other loci. However, increased expression of other oncogenes, i.e., Ha-*ras*, N-*myc*, c-*myc*, and c-*raf*, was not detected. TBE-1 and TBE-1SA acquired a general resistance to differentiation inducers, i.e., serum and TPA, indicating an imbalance in their growth and differentiation program.

We have also transfected NHBE cells with the gene from a Burkitt's lymphoma cell line (B-*myc*) by protoplast fushion technique. The B-*myc* transfected cells were selected in 4% serum or TPA (10 nM) and showed a 5-fold increase in the frequency of differentiation-resistant colonies compared to the control. The cells of the resistant colonies had a finite lifespan. Therefore, an SV40 T-antigen immortalized cell line (BEAS-2B) appeared to be a more suitable recipient to study the effects of oncogenes in NHBE cells. The introduction of the lung cancer-associated oncogenes *ras*, *myc*, and *raf* was performed by infection or transfection (Table 3). The v-Ha-*ras* retroviral recombinant was introduced into BEAS-2B (*127*), whereas c-*myc* and c-*raf* sequences were transfected via strontium phosphate precipitation (*110*). The infected or transfected BEAS-2B underwent a selection for geneticin resistance prior to inoculation into nude mice, with the exception of Ki-*ras* infected cells. v-Ha-*ras* or v-Ki-*ras* gave rise to tumors in a high frequency (Table 3) within 3 to 6 weeks with the following characteristics: very similar morphology, transplantability, human origin, anaplastic carcinomas, increased ploidy and expression of T-antigen (*127*).

Interestingly, both the v-Ha-*ras* infectants and tumor cell lines established from v-Ha-*ras*- or v-Ki-*ras*-induced tumors were not only resistant to but were stimulated by serum, a characteristic that had been previously shown for cells isolated from human tumors. c-*raf* or c-*myc* alone were not sufficient to induce tumors in nude mice, whereas the combination of c-*raf* and c-*myc* was tumorigenic with an incidence of 5/8 animals (A. M. A. Pfeifer, G. E. Mark, and C. C. Harris, unpublished data, Table 3). These tumors were classified as undifferentiated carcinomas and were of human origin. Experiments examining the response of c-*raf*/c-*myc* tumor cells to inducers of squamous differentiation are being performed.

Our data to date strongly support the hypothesis that uncoupling of terminal squamous differentiation and proliferation pathways are involved in the generation of neoplasms. The tumorigenic BEAS-2B transfectants tested acquire resistance to differentiation inducers. However, loss of response to differentiation-inducing agents is

Table 3. Effects of (proto)oncogenes on tumorigenicity of T-antigen gene containing human bronchial epithelial cells (BEAS-2B).

Plasmid	(Proto)oncogene	Mode	Tumorigenicity	Latency, weeks
Zip neo	—	Transfection/infection	0/22	NA
Zip neo-v-Ha-*ras*[a]	v-Ha-*ras*	Infection	12/15	3
KiSV	v-Ki-*ras*	Infection	4/7	6
Zip neo-c-*myc*	c-*myc*	Transfection	0/10	NA
Zip neo-c-*raf*	c-*raf*	Transfection	0/10	NA
Zip neo-c-*myc*	c-*myc*	Transfection	5/8	4
Zip neo-c-*raf*	c-*raf*			

[a]The infection of BEAS-2B with the Zip neo-v-Ha-*ras* recombinant virus has been described elsewhere (*127*). The Ki-*ras* retrovirus required baboon endogenous virus as a helper cell line. The Zip neo-c-*myc* plasmid consisted of a full length murine c-DNA cloned into Zip neo SV(X) (*31*). Zip neo-c-*raf* was generated by cloning the full length c-DNA of the c-*raf* gene resected at the 5′ end by 39 nucleotides into Zip neoSV(X) (*126*, A. M. A. Pfeifer, G. E. Mark, C. C. Harris, unpublished data).

insufficient for tumorigenicity as serum-resistant subclones of BEAS-2B have not as yet formed tumors in nude mice (K. Yang, J. F. Lechner, and C. C. Harris, unpublished observation). This suggests that aberrations in the pathways of differentiation are cooperating with other events but are insufficient to cause tumorigenesis.

The physiological function of the *ras* gene family is different from that of *raf* and *myc*, suggesting a dissimilar modus for transformation. The functional and biochemical homologies between $p21^{ras}$ protein and G proteins argues that p21 protein is involved in signal transduction, and it has been shown that transforming p21 has very rapid effects on the phosphoinositol turnover in some cell types (*128*). Constitutive activation of *ras* protein caused by loss of GTPase activity, increased association of transformed p21 and GTP to the biological active form or transformational change of the protein with loss of GTP-binding requirement for activation may result in continuous signal flow and subsequently to transformation (*129*).

A cooperative effect of the *raf* and *myc* oncogenes has been described in other systems. Both v-*myc* and v-*raf* genes must be expressed to induce continuous proliferation of murine bone marrow cells (*130*). Infection of interleukin-3 (IL-3) responsive murine bone marrow cells with v-*raf* induced their continuous *in vitro* proliferation, whereas v-*myc* infection caused independence of the IL-3 requirement (*131*). It may be speculated that v-*myc* reprograms the cells, whereas v-*raf* acts as a primary oncogene that efficiently induces the cells to proliferate. This hypothesis is consistent with the observation that tumor cell lines established from *raf*/*myc* transfected BEAS-2B cells require conditioned medium for sufficient growth, suggesting the secretion of an autocrine factor. In this context, it has been shown that the introduction of *ras* or *myc* oncogenes into various cell types results in the secretion of TGF-α (*132*,*133*) and that expression of TGF-α induces transformation of rat fibroblasts (*134*). In addition, many human small cell lung carcinoma lines with *myc* amplification and *raf* over-expression produce the mitogen bombesin, which is growth stimulating for these tumor cells and also for NHBE cells (*135*). Further studies are required to determine the biochemical and biological character of the factor(s) released by the NHBE transfectants and their correlation to transformation. It also remains to be clarified if the presence of SV40 T-antigen sequences can modulate effects of the transfected oncogenes and if the results of our transformation studies on BEAS-2B cells can be generalized for epithelial cells derived from different donors.

Conclusion

The multistage process of carcinogenesis is often associated with altered expression of differentiated functions, and we have found that oncogenic agents can cause disorders of mechanisms regulating terminal differentiation. We have shown that serum, TGF-β_1, tumor promoters, tobacco smoke condensate, and aldehydes induce squamous differentiation of NHBE cells. The differentiation process can be dissociated from direct effects of cAMP and free cytosolic Ca^{2+} except for aldehydes, where aldehyde-induced elevation of free cytosolic Ca^{2+} may be involved in the formation of cross-linked envelopes. Induction of squamous differentiation is accompanied by a rapid induction of c-*fos* and reduction of c-*myc* steady state in mRNA levels, suggesting a role of these protooncogenes for signal transduction. In contrast to normal cells, human lung carcinoma cell lines are less responsive to induction of squamous differentiation by TGF-β_1 or TPA, and this decreased responsiveness is not due to changes of receptor affinity or number. Carcinogenesis studies with nickel sulfate support the hypothesis that inhibition of differentiation processes is associated with abberations in proliferation pathways. The introduction of v-Ha-*ras* or v-Ki-*ras* oncogenes and the combination of c-*raf* and c-*myc* protooncogenes result in transformation of NHBE cells or SV40 T antigen immortalized BEAS-2B cells. The associated resistance to differentiation inducers appears to be a necessary but insufficient step in the transformation process. Moreover, it may confer a selected clonal expansion advantage to initiated cells. The elucidation of the physiological role of protooncogenes including their potential function as growth factors or growth factor activating agents will help to understand the interactions between pathways controlling differentiation and proliferation.

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